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16. ABSTRACT

Formaldehyde is a large production volume chemical widely distributed in research laboratories, industrial workplaces, and home and personal environments. Inhalation studies with formaldehyde have documented its ability to produce squamous cell carcinomas in rats. When primary hamster embryo cells were treated by gaseous exposure to formaldehyde or by incorporation into the medium, a dose-related increase in the frequency of SA7 virus transformation was produced. The length of chemical treatment and the time interval before subsequent addition of transforming virus was critical, with two-hr treatment times as the most efficient. Treatment by gaseous exposure permitted utilization of lower treatment concentrations. Determination of formaldehyde concentrations in culture media of bioassay dishes treated by this method documented that 2.2 μg/ml produced significantly enhanced viral transformation. Exposure of hamster embryo cells to formaldehyde by these methods produces reproducible and quantitative genotoxic effects.

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Synergism in the Transformation of Hamster Embryo Cells Treated With Formaldehyde and Adenovirus

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Formaldehyde is a large production volume chemical widely distributed in research laboratories, industrial workplaces, and home and personal environments. Inhalation studies with formaldehyde have documented its ability to produce squamous cell carcinomas in rats. When primary hamster embryo cells were treated by gaseous exposure to formaldehyde or by incorporation into the medium, a dose-related increase in the frequency of SA7 virus transformation was produced. The length of chemical treatment and the time interval before subsequent addition of transforming virus was critical, with two-hr treatment times as the most efficient. Treatment by gaseous exposure permitted utilization of lower treatment concentrations. Determination of formaldehyde concentrations in culture media of bioassay dishes treated by this method documented that 2.2 μg/ml produced significantly enhanced viral transformation. Exposure of hamster embryo cells to formaldehyde by these methods produces reproducible and quantitative genotoxic effects.

Key words: formaldehyde, viral transformation, Syrian hamster embryo cells

INTRODUCTION

Industrial, occupational, and personal exposures to formaldehyde are of concern because of their widespread nature, relatively high exposure levels, and high chemical reactivity of the compound.

Formaldehyde is a commodity chemical with worldwide production approaching 12 million tons [Gerberich et al, 1980]. Examples of its diverse industrial uses range from its addition to wood products, insulating, and molding materials to incorporation

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into soaps, shampoos, and textile materials [Gerberich et al, 1980]. Agricultural uses include its incorporation into fertilizers, disinfectants, insecticides, and fungicides [Auerbach et al, 1977]. Formaldehyde is utilized as a fixative and inactivating agent in research and medical laboratories [Auerbach et al, 1977]. It is present in automobile, diesel, and incinerator exhausts as well as in tobacco smoke [Altshuller et al, 1963; Kensler and Battista, 1963]. Cigarette smoke may contain as much as 40 ppm formaldehyde [Kensler and Battista, 1963]. Formaldehyde has been detected in photochemical smog at levels up to 0.16 ppm [Altshuller et al, 1963] and between 0.03 to 8.5 ppm in some mobile and stationary homes insulated with urea formaldehyde or constructed with particle board [Breysse, 1977; Sardinas et al, 1979].

Formaldehyde has been documented to produce genotoxic effects in both prokaryotic and eukaryotic organisms by its ability to react freely with amino groups on nucleic acids, proteins, or their precursors [Auerbach et al, 1977]. Formaldehyde induces unscheduled DNA synthesis in HeLa cells [Martin et al, 1978] and causes protein-DNA and DNA-DNA cross links in both E coli [Wilkins and MacLeod, 1976] and mammalian cells treated in culture [Ross and Shipley, 1980; Swenberg et al, 1980]. Sister chromatid exchanges were reported in mice treated with formaldehyde [Brusick et al, 1980].

Chronic exposure of rats and mice to formaldehyde vapor (14–15 ppm) produced squamous cell carcinomas of the nasal cavity [Boreiko et al, 1982; Albert et al, 1982; Swenberg et al, 1980]. In culture, formaldehyde has been reported to be an initiating agent in C3H/10T½ mouse embryo cells at concentrations of 0.5–1.0 μg/ml [Ragan and Boreiko, 1981].

In the environment, formaldehyde exposure occurs commonly in the form of a vapor and may be combined in a complex mixture containing other recognized or putative carcinogens and/or mutagens. Its presence in gasoline and diesel exhausts, cigarette smoke, and incinerator wastes are prime examples. The possibility of additive or synergistic genotoxic action with other chemicals, with formaldehyde itself, and with transient or indigenous viruses is a logical but untested possibility. Rats treated with formaldehyde and subsequently displaying nasal cell carcinomas [Swenberg et al, 1980] were reported to carry an enzootic agent (sialodacryoadenitis virus).

Methods for treating cultured cells with gases or vapors of volatilized liquids have been developed in our laboratory [Hatch et al, 1981, 1982]. A number of volatile and gaseous chlorinated methanes and ethanes have been evaluated using this exposure methodology for their ability to enhance viral transformation in Syrian hamster embryo cells [Hatch et al, 1981, 1982].

This report describes the treatment of hamster cells in culture with formaldehyde in the vapor state and in solution and the increased viral transformation observed following addition of simian adenovirus, SA7. The length of chemical treatment and the time interval before subsequent addition of transforming virus was investigated.

MATERIALS AND METHODS

Cell Cultures

Primary Syrian hamster embryo cells (HEC) were prepared by trypsinization of eviscerated and decapitated embryos (Charles River Laboratories, Boston, Massachusetts) after 13–14 days gestation. Cells were resuspended in modified Dulbecco's

medium (MDM) [Casto, 1973] supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sterile Systems, Inc., Logan, Utah) and $NaHCO_3$ (0.22 g/ 100 ml). Approximately 5×10^6 cells in 5 ml of medium were plated into 60 mm plastic Petri dishes and incubated in a 5% CO_2 atmosphere at 37°C. Total cell number after three days ranged from 4 to 6×10^6 cells/plate.

Continuous lines of Vero monkey kidney cells (ATCC:CC1-81) were subcultured weekly in 100-mm plastic Petri dishes. Growth medium consisted of Eagle's minimum essential medium with 10% heat-inactivated FBS.

Virus

Simian adenovirus SA7 was inoculated into Vero cell cultures in 100-mm dishes at an input multiplicity of two to three plaque-forming units per cell. After the virus had been adsorbed for 2 hr, 5 ml of medium were added to each plate. Cytopathic effects were usually complete by 72 hr, after which the infected cells were harvested, frozen-thawed four times, and the virus separated from cell debris by low-speed centrifugation. Virus stocks for transformation assays were stored in 1- or 2-ml aliquots at −65°C.

Treatment With Formaldehyde Vapor

Cells were cultured in 60-mm glass (Corning Pyrex) Petri dishes. Fresh medium was added to the cells 8–24 hr before treatment; this medium was replaced prior to treatment with 1 ml of medium supplemented with 7.5 mM HEPES (N-2-hydroxyethyl piperazine) buffer in place of sodium bicarbonate. Commercially available polycarbonate treatment chambers of 4.6 liter volume (Billups-Rothenberg, Flow Labs, McLean, Virginia) were used. Ten ml of sterile water were added to the bottom of each chamber to ensure adequate humidification and to prevent desiccation of the small volume of cell culture medium. Formaldehyde solution (37%, Fisher Scientific) was diluted in sterile deionized distilled water and mechanically dispensed with a glass pipette into an open glass Petri dish. The dishes were quickly placed into the exposure chambers containing dishes of cells, and the chambers were rapidly sealed.

Following introduction of formaldehyde, the sealed chambers and dishes of cells were placed into a rocker platform and incubated at 37°C. The chambers were tilted to provide a sufficient inclination (10°) and rocked four times per min, so that a portion of the cell monolayer was always in contact with the formaldehyde vapors. Treatment times were 2 or 20 hr, after which the vapor was exhausted and the cells assayed for viability and enhancement of viral transformation. The concentration of formaldehyde in the media after exposure was determined according to Nash [1953].

Treatment With Formaldehyde in Culture Media

The HEC cells were treated with formaldehyde by adding appropriate dilutions of a 37% stock to the culture media. Treatment times were either 2 or 20 hr, after which the cells were rinsed with complete medium and inoculated with SA7.

Transformation Assays

Adenovirus transformation procedures have been presented elsewhere [Casto, 1968, 1973]. Briefly, the procedure was as follows. SA7 was added to HEC (3 to 4 $\times 10^7$ plaque-forming units per culture dish containing 5–7 $\times 10^6$ cells/60-mm dish) and adsorbed for 3 hr; the virus-inoculated cells were removed with trypsin (0.25%

trypsin in MDM with 0.1 mM $CaCl_2$), centrifuged, and resuspended to 10^6 cells/ml in MDM with 10% FBS and $NaHCO_3$ (0.11g/100 ml). The cells were mixed vigorously and plated into 60-mm dishes using 2×10^5 cells/dish; 3 ml of the above medium were then added to each plate. After incubation for 3 days, the medium was changed to MDM with 0.1 mM $CaCl_2$ [Casto, 1968; Freeman et al, 1967], 10% FBS, and $NaHCO_3$ (0.22g/100 ml). After six days, transformation assay plates were overlaid with 3 ml of the above medium containing Bacto-agar (0.3g/100 ml). At intervals of 4, 5, and 6 days, 3 ml of additional agar medium were added. Final focus counts were made 25 to 30 days from the beginning of the experiment. Plates were fixed with buffered formalin and stained with Giemsa.

Survival Assays

After resuspension of the HEC to 10^6 cells/ml (see Transformation Assays), the cells were diluted 1:300 in medium to give 333 cells/0.1 ml. Two-tenths ml (666 cells) were then added to each of five plastic dishes, followed by 3 ml of MDM with 10% FBS and $NaHCO_3$ (0.11g/ml). Five to six days later, 3 ml of medium with double the amount of $NaHCO_3$ were added to each plate, and after eight to nine days of total incubation, the cell colonies were fixed in 10% buffered formalin and stained with 0.02% crystal violet. The cloning efficiency of virus-inoculated cells under these conditions was usually 8 to 15%. The number of colonies in five plates seeded with treated cells was divided by the number of colonies in five plates with control cells to give the surviving fraction of treated cells.

Determination of Enhancement

The total number of SA7 foci in five control plates, each receiving 2×10^5 cells, was used to determine the frequency of transformation per 10^6 virus-inoculated control cells. The transformation frequency (TF) of treated cells (number of SA7 foci per 10^6 surviving cells) was calculated by multiplying the number of SA7 foci from five plates by the reciprocal of the surviving fraction of treated cells. Detailed methods for determining the frequency of transformation and enhancement ratios using data from actual enhancement experiments have been published [Casto et al, 1974, 1976]. Similar methods for calculation have been used for the determination of mutation frequency [Chu, 1971] and for the demonstration of enhancement of SV40 transformation by DNA base analogs [Todaro and Green, 1964] in mammalian cells.

Enhancement was expressed as the ratio between the TF of treated surviving cells and the TF of control cells. Statistical significance was determined using a table of ratios [Casto et al, 1978] derived from the Lorenz table [Lorenz, 1962] based upon the Poisson distribution. The increased TF was considered statistically significant at the 5 or 1% confidence level if the enhancement ratio exceeded the appropriate value obtained from the Lorenz table.

RESULTS

The effect of pretreatment for 20 hr of mass culture of primary hamster embryo cells with the vapor phase of formaldehyde in closed treatment chambers is shown in Table I. Formaldehyde was bioassayed over a 20-fold range. This treatment regimen produced toxic to nontoxic responses as evaluated by survivors in plating efficiency assays. Overt toxicity was observed within a narrow concentration range. No signifi-

TABLE I. Enhancement of SA7 Transformation by Formaldehyde: Vapor Phase Treament for 20 hr

Treatment dose (μl/l)[a]	Toxicity (%)[b]	SA7 foci[c]	Enhancement ratio[d]
2	100	0	NA[e]
1	0	136	1.2
0.5	18	100	1.1
0.3	0	67	0.6
0.1	0	89	0.8
0	0	109	1.0

[a]μl/l = μl of formaldehyde/liter chamber volume.
[b]Determined from plates receiving 666 cells. Cloning efficiency of control cells was from 8 to 15%.
[c]Number of foci from 2×10^6 plated cells.
[d]Enhancement ratio was determined by dividing the transformation frequency (TF) of treated cells (TF = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Data are from one of three replicate experiments, each of which gave similar results.
[e]NA = not applicable.

TABLE II. Enhancement of SA7 Transformation by Formaldehyde: Vapor Phase Treatment for 2 hr

Treatment dose (μl/l)[a]	Concentration after exposure (μg/ml)[b]	Toxicity (%)[c]	SA7 foci[d]	Enhancement ratio[e]
6.0	23.3	63	196	**13.1**
3.0	—	0	85	**2.1**
1.5	2.2	0	97	**2.4**
0.8	—	0	97	**2.4**
0.4	0.35	0	54	1.4
0	0.00	0	40	1.0

[a]μl/l = μl of formaldehyde/liter chamber volume.
[b]Values are the means of six replicate determinations of formaldehyde concentration in medium. The coefficient of variation was 20%.
[c]Determined from plates receiving 666 cells. Cloning efficiency of control cells was from 8 to 15%.
[d]Number of foci from 2×10^6 plated cells.
[e]Enhancement ratio was determined by dividing the transformation frequency (TF) of treated cells (TF = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Bold numbers indicate significance at the 1% level. Data are from one of three replicate experiments, each of which gave similar results.

cant increase in viral transformation occurred following 20 hr of formaldehyde pretreatment.

The effect of pretreatment cells with formaldehyde for 2 hr is described in Table II. A threefold increase in the highest exposure dose (6 μl/l versus 2 μl/l*) was needed to produce overt cytotoxicity in 2 versus 20 hr treatment. Pretreatment with

*μl/l = μl of formaldehyde per liter of treatment chamber volume.

formaldehyde for 2 hr followed by the immediate addition of virus produced a concentration-dependent significant increase in the transformation frequency. An absolute increase in number of viral foci was produced at four exposure levels tested (ie, 6, 3, 1.5, and 0.8 μl/l). Therefore, the increased enhancement ratios were not dependent on cell lethality for statistical significance.

Table III shows the effect of formaldehyde pretreatment for 2 hr followed by a 20 hr recovery period before addition of virus to treated cells. Severe cell killing was noted at the 6 μl/l exposure dose, but the general cytotoxic response was as observed in Table II. An enhancement of viral transformation was seen only at the highest treatment dose (6 μl/l). However, there was no absolute increase in virally induced transformed foci, therefore, the enhancement ratio was dependent upon correction for cell lethality to achieve statistical significance. It appears that the toxic effects persist through the 20 hr recovery period, but significant enhancement of viral transformation does not. It is clear that the length of chemical treatment and the time interval before subsequent addition of SA7 virus are critical for demonstrating an increased viral transformation frequency with formaldehyde treatment.

When formaldehyde was administered to HEC for 2 hr by direct addition to the culture media cytotoxicity was evident at relatively high concentrations (62–125 μg/ml). These treatment concentrations produced significant enhancement of SA7 viral transformation with absolute increases in the number of viral foci/dish (Table IV). Similar experiments using a 20 hr treatment prior to virus addition produced cytotoxicity at equivalent concentrations but no viral enhancement. (Data not shown).

DISCUSSION

The enhancement of viral transformation by physical or chemical agents has been successfully employed in mammalian cells to study the interactions between viral and chemical carcinogens. Experimental data suggest that discrete changes in cell DNA resulting from chemical treatment can be detected by observing a quantitative increase in viral transformation. Increases in viral transformation have been demonstrated in hamster, rat and human cells treated with a variety of carcinogenic agents [Casto, 1981]. The viral enhancement assay reflects the capacity of a chemical to damage cell DNA by either direct or indirect means. It has been shown that carcinogen treatment results in an increased incorporation of viral DNA into cellular DNA [Casto et al, 1979]. Data from the bioassay of approximately 130 test chemicals from 29 different chemicals classes using the enhancement of viral transformation assay have been reported [Casto, 1981a, b; Hatch et al, 1981, 1982].

In this report, we document the successful use of the viral enhancement assay with hamster embryo cells and formaldehyde. Enhanced viral transformation was dependent upon the length of chemical treatment and/or the time of subsequent addition of transforming virus.

The success of both a short treatment time and immediate addition of virus probably reflects (Table II) the nature of the chemical action of formaldehyde with DNA and the proposed mechanism for enhanced viral transformation. Formaldehyde is presumed to be a direct acting agent similar to radiation or alkylating agents. Formaldehyde-induced lesions are subject to repair in microorganisms [Auerbach et al, 1977], and formaldehyde has been shown to induce unscheduled DNA synthesis in HeLa cells [Martin et al, 1978].

The longer treatment time (20 hr) may not be as efficient since the effective formaldehyde vapor concentrations probably decrease with time, presumably by adsorption and/or penetration of chamber walls, culture media, and cells. Such a decrease in chemical vapor concentrations in these treatment chambers has been documented by chemical analysis of volatile or gaseous chlorinated compounds of equivalent volatility as formaldehyde over increasing time as determined by gas chromatographic techniques [Hatch et al, 1981, 1982].

TABLE III. Enhancement of SA7 Transformation by Formaldehyde: Vapor Phase Treatment for 2 hr*

Treatment dose (μl/l)[a]	Toxicity (%)[b]	SA7 foci[c]	Enhancement ratio[d]
6.0	96	12	**7.9**
3.0	11	56	*1.7*
1.5	14	40	1.2
0.8	2	40	1.1
0.4	0	45	1.2
0	0	38	1.0

*Virus added 20 hr post chemical exposure.
[a]μl/l = μl of formaldehyde/liter chamber volume.
[b]Determined from plates receiving 666 cells. Cloning efficiency of control cells was from 8 to 15%.
[c]Number of foci from 2×10^5 plated cells.
[d]Enhancement ratio was determined by dividing the transformation frequency (TF) of treated cells (TF = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Bold type numbers are significant at the 1% level; italic numbers at the 5% level. Data are from one of three replicate experiments, each of which gave similar results.

TABLE IV. Enhancement of SA7 Transformation of Syrian Hamster Embryo Cells by Pretreatment With Formaldehyde in Medium for 2 hr

Treatment dose (μg/ml)[a]	Toxicity (%)[b]	SA7 foci[c]	Enhancement ratio[d]
125	90	89	**23.4**
62	48	151	**7.6**
31	1	46	1.2
16	5	37	1.0
0	0	38	1.0

[a]Formaldehyde was dissolved in medium and dilutions were added to mass cultures of HEC cells.
[b]Determined from plates receiving 666 cells. Cloning efficiency of control cells was from 8 to 15%.
[c]Number of foci from 2×10^5 plated cells.
[d]Enhancement ratio was determined by the transformation frequency (TF) of treated cells (T = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Bold numbers indicate significance at the 1% level. Data are from one of three replicate experiments, each of which gave similar results.

If the levels of formaldehyde decrease with time to ineffective levels, then a longer treatment time might provide a period for cellular repair processes to remove DNA damage. This hypothesis is supported by the observation that enhancement does not occur if a 20 hr recovery period (before addition of virus) is allowed following a 2 hr treatment time or if treatment is extended to 20 hr. A correlation between enhancement of viral transformation and persistence of DNA damage by both alkylating agents and polycyclic hydrocarbons has been demonstrated [Casto et al, 1976].

The increased viral transformation in gaseous exposed cells seem to require lower concentrations of formaldehyde than cells treated with formaldehyde dissolved in culture media. This observation is consistent with previously reported results with dichloromethane and 1,1,1-trichloroethane [Hatch et al, 1982]. The reasons for this increased sensitivity are not understood, but may be related to the extent of solvation of these chemicals in solution compared to the vapor state. This may have an effect on cell permeability. The use of vapor phase treatment allows utilization of lower treatment doses. This should reduce the cytotoxic effects of chemicals, thus permitting increased detection of genotoxic events.

CONCLUSIONS

Hamster embryo cells treated by exposure to formaldehyde demonstrate a dose related increase in the frequency of SA7 virus transformation. The length of treatment time and the time interval before subsequent bioassay are critical for optimal activity. Cells treated with formaldehyde in the vapor state exhibited enhanced viral transformation at lower concentrations than those exposed to formaldehyde added to culture media.

The use of enhanced viral transformation offers a model system for evaluating the potential additive or synergistic genotoxic effects of formaldehyde with other chemicals, chronic exposure to formaldehyde itself, or interaction with transient or indigenous viruses. These are important parameters to examine due to its ubiquitous nature and the complex mixtures in which formaldehyde vapor is presented to human populations.

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